IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of	)	Examiner: Goddard L.
	)	
Surinder K. Batra et al.	)	Art Unit: 1642
	)	
Serial No.: 10/721,553	)	Response to Paper No. n/a
	)	
Filed: November 25, 2003	)	
	)	
For: "ANTIBODIES IMMUNOLOGICALLY SPECIFIC FOR PD2, A PROTEIN THAT IS AMPLIFIED AND OVEREXPRESSED IN PROSTATE CANCER"	)	

DECLARATION OF SURINDER K. BATRA

AND MICHAEL A. HOLLINGSWORTH UNDER 37 C.F.R. §1.131

We, Surinder K. Batra and Michael A. Hollingsworth, hereby declare that:

I. We are the co-inventors of the invention described and claimed in U.S. Patent Application Serial No. 10/721,553, (the '553 application).

II. We have read and are familiar with the contents of the Official Action dated October 25, 2005 in the '553 application. We note that the Examiner has rejected claims 28-33 under 35 U.S.C. §102(e) as allegedly anticipated by Lal et al. US Patent No. 5,932,442 (the '442 patent). The Examiner also rejected claims 34-36 under 35 U.S.C. §103(a)

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over the '442 patent in view of Sigma Immuno Chemicals 1993 Catalog. The purpose of this declaration is to establish that the invention described and claimed in the '553 application (the "subject invention") was completed prior to September 23, 1997, which upon information and belief, is the earliest U.S. filing date of '442 patent.

III. Conception of the invention described in the present application and the reduction to practice thereof is evidenced by copies of a National Institutes of Health (NIH) grant proposal submitted by us attached hereto as Exhibit A. At pages 28-33 the sequences of the PD2 cDNA and PD2 protein are provided. Also disclosed is the *in vitro* translational product of PD2 cDNA, chromosomal mapping of the PD2 gene, and the differential expression of PD2 in pancreatic tumor cell lines representing various morphological stages of pancreatic cell differentiation. The grant proposal describes at page 36 an antibody raised against PD2 Peptide 2 (amino acids 327-348 of SEQ ID NO: 2). This antibody was used in Western blots to detect PD2 protein expression levels.

IV. Attached hereto as Exhibit B are pages of the NIH issued grant proposal receipt. The dates on the receipt have been masked for the purpose of this Declaration.

V. The experiments described in Exhibit A were performed prior to the U.S. filing date of '442 patent.

VI. We were in possession of the invention claimed in the '553 application at least as early as June of 1997. The

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invention was conceived of, and reduced to practice in the United States of America.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the above-referenced application or any patent issued thereon.

1/20/2006
DATE

Surinder K. Batra

DATE

Michael A. Hollingsworth

Enclosure: Exhibit A
Exhibit B

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JAN 27 2006
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We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the above-referenced application or any patent issued thereon.

DATE

Surinder K. Batra

1/25/06

DATE

Michael A. Hollingsworth

Enclosure: Exhibit A
Exhibit B

EXHIBIT
A
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3. Preliminary Data

3.1. Isolation of PD2 cDNA

A cDNA library from a poorly differentiated human pancreatic tumor cell line, Panc 1, was screened for differentially expressed mRNAs using single stranded cDNA probes synthesized from mRNA from the well-differentiated CD-11 (Kim *et al.*, 1989) and poorly differentiated Panc 1 (Liber *et al.*, 1975) human pancreatic tumor cell lines. Seventeen clones were obtained that hybridized very strongly to the Panc 1 probe and did not hybridize to CD-11 probe. The characterization of two of these clones has previously been published: one encoded the human ribosomal protein S16 (Batra *et al.*, 1991b), and the other (we named PD-1) encoded the human ribosomal protein rpL17 (Batra *et al.*, 1991c). The cDNA reported here in this proposal we named pancreatic differentiation 2 (PD2). A representative Northern blot in which the PD2 cDNA insert hybridized to a mRNA transcript expressed at approximately 30-fold higher levels in Panc 1 as compared with CD-11, as determined by densitometric analysis is shown in Figure 1A. The detected transcript size and isolated insert size (1.9 Kb) suggest that the cDNA is close to full length. The same filter was probed with a human β-actin cDNA (Gunning *et al.*, 1987) as a control for the quality and quantity of mRNA (Figure 1B).

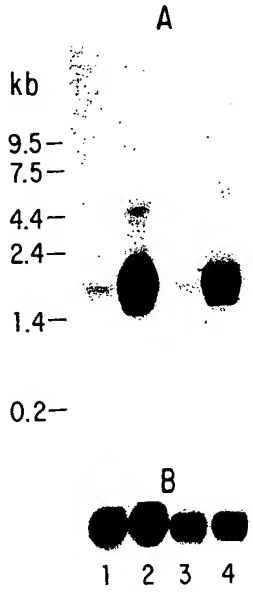

Figure 1. Northern blot analysis of PD2 cDNA probe with RNA from differentiated and undifferentiated pancreatic cell lines. *Lanes 1 and 2 contained total RNA from CD-11 and Panc 1, respectively. Lanes 3 and 4 contained poly(A+) RNA from CD-11 and Panc 1, respectively. The blot was hybridized to PD2 (A) and actin (B).*

To obtain additional clones of the normal PD2 cDNA, we screened a normal human pancreatic cDNA library using PD2 probe derived from Panc1, and isolated five different cDNA clones of various insert sizes.

3.2. Sequence of the PD2 cDNA

All clones isolated from Panc1 tumor cell line and from a normal pancreatic cDNA library showed 100% sequence identity. The complete nucleotide sequence and the deduced amino acid sequence of the longest PD2 cDNA are shown below in Figure 2. The DNA sequence contained a 5' untranslated region of 156 bp. and a 3' untranslated region of 138 bp. The non-coding 3' region contained the polyadenylation signal AATAAA as underlined in Figure 2. An open reading frame from 157 to 1752 yields a predicted translation product of 60 kDa.

```
1       TTCTCGCCCGCCCACCTCATCTCAACCCACTTTCCGCGGGGAGCGGCGCCAAGCTGGGCC
61      TTCCTCGGATCAGGCGTCCCCTGAAGTCGGCACGCCCCTCTGCGTCCCCCTTCGGTCCCG
121     CTAGGACCCCGTCCGGGCTGCCGTCGCCTCGTCGCTATGGCGCCCACCATCCAGACCCAG
                                            M  A  P  T  I  Q  T  Q    8
181     GCCCAGCGGGAGGATGGCCACAGGCCCAATTCCCACCGGACTCTGCCTGAGAGGTCTGGA
        A  Q  R  E  D  G  H  R  P  N  S  H  R  T  L  P  E  R  S  G   28
241     GTGGTCTGCCGAGTCAAGTACTGCAATAGCCTCCCTGATATCCCCTTCGACCCCAAGTTC
        V  V  C  R  V  K  Y  C  N  S  L  P  D  I  P  F  D  P  K  F   48
301     ATCACCTACCCCTTCGACCAGAACAGGTTCGTCCAGTACAAAGCCACTTCCTTGGAGAAA
        I  T  Y  P  F  D  Q  N  R  F  V  Q  Y  K  A  T  S  L  E  K   68
361     CAGCACAAACATGACCTCCTGACTGAGCCAGACCTGGGGGTCACCATCGATCTCATCAAT
        Q  H  K  H  D  L  L  T  E  P  D  L  G  V  T  I  D  L  I  N   88
420     CCTGACACCTACCGCATCGACCCCAATGTTCTTCTAGATCCAGCTGATGAGAAACTTTTG
        P  D  T  Y  R  I  D  P  N  V  L  L  D  P  A  D  E  K  L  L  108
481     GAAGAGGAGATTCAGGCCCCCACCAGCTCCAAGAGATCCCAGCAGCACGCGAAGGTGGTG
        E  E  E  I  Q  A  P  T  S  S  K  R  S  Q  Q  H  A  K  V  V  128
541     CCATGGATGCGAAAGACAGAGTACATCTCCACTGAGTTCAACCGTTATGGCATCTCCAAT
        P  W  M  R  K  T  E  Y  I  S  T  E  F  N  R  Y  G  I  S  N  148
601     GAGAAGCCTGAGGTCAAGATTGGGGTTTCTGTGAAGCAGCAGTTTACCGAGGAAGAAATA
        E  K  P  E  V  K  I  G  V  S  V  K  Q  Q  F  T  E  E  E  I  168
661     TACAAAGACAGGGATAGCCAGATCACAGCCATTGAGAAGACTTTTGAGGATGCCCAGAAA
        Y  K  D  R  D  S  Q  I  T  A  I  E  K  T  F  E  D  A  Q  K  188
721     TCAATCTCACAGCATTACAGCAAACCCCGAGTCACACCGGTGGAGGTCATGCCTGTCTTC
        S  I  S  Q  H  Y  S  K  P  R  V  T  P  V  E  V  M  P  V  F  208
781     CCAGACTTTAAGATGTGGATCAATCCATGTGCTCAGGTGATCTTTGACTCAGACCCAGCC
        P  D  F  K  M  W  I  N  P  C  A  Q  V  I  F  D  S  D  P  A  228
841     CCCAAGGACACGAGTGGTGCAGCTGCGTTGGAGATGATGTCTCAGGCCATGATTAGGGGC
        P  K  D  T  S  G  A  A  A  L  E  M  M  S  Q  A  M  I  R  G  248
901     ATGATGGATGAGGAAGGGAACCAGTTTGTGGCCTATTTCCTGCCTGTAGAAGAGACGTTG
        M  M  D  E  E  G  N  Q  F  V  A  Y  F  L  P  V  E  E  T  L  268
961     AAGAAACGAAAGCGGGACCAGGAGGAGGAGATGGACTATGCACCAGATGATGTGTATGAC
        K  K  R  K  R  D  Q  E  E  E  M  D  Y  A  P  D  D  V  Y  D  288
1021    TACAAAATTGCTCGGGAGTACAACTGGAACGTGAAGAACAAAGCTAGCAAGGGCTATGAG
        Y  K  I  A  R  E  Y  N  W  N  V  K  N  K  A  S  K  G  Y  E  308
1081    GAAAACTACTTCTTCATCTTCCGAGAGGGTGACGGGGTTTACTACAATGAGTTGGAAACC
        E  N  Y  F  F  I  F  R  E  G  D  G  V  Y  Y  N  E  L  E  T  328
1141    AGGGTCCGCCTTAGTAAGCGCCGGGCCAAGGCTGGGGTTCAGTCAGGCACCAACGCCCTG
        R  V  R  L  S  K  R  R  A  K  A  G  V  Q  S  G  T  N  A  L  348
1201    CTTGTGGTCAAACATCGGGACATGAATGAGAAGGAACTGGAAGCTCAGGAGGCACGGAAG
        L  V  V  K  H  R  D  M  N  E  K  E  L  E  A  Q  E  A  R  K  368
1261    GCCCAGCTAGAAAACCACGAACCGGAGGAGGAAGAGGAAGAGGAGATGGAGACAGAAGAG
        A  Q  L  E  N  H  E  P  E  E  E  E  E  E  E  M  E  T  E  E  388
1321    AAAGAAGCTGGGGGCTCAGATGAGGAGCAGGAGAAGGGCAGCAGCAGTGAGAAGGAGGGC
        K  E  A  G  G  S  D  E  E  Q  E  K  G  S  S  S  E  K  E  G  408
1381    AGTGAAGATGAGCACTCGGGCAGCGAGAGTGAACGGGAGGAAGGTGACAGGGACGAGGCC
        S  E  D  E  H  S  G  S  E  S  E  R  E  E  G  D  R  D  E  A  428
1441    AGTGACAAGAGTGGCAGTGGTGAGGACGAGAGCAGCGAGGATGAGGCCCGGGCTGCCCGT
        S  D  K  S  G  S  G  E  D  E  S  S  E  D  E  A  R  A  A  R  448
1501    GACAAAGAGGAGATCTTTGGCAGTGATGCTGATTCTGAGGACGATGCCGACTCTGATGAT
        D  K  E  E  I  F  G  S  D  A  D  S  E  D  D  A  D  S  D  D  468
1561    GAGGACAGAGGACAGGCCCAAGGTGGCAGTGACAATGATTCAGACAGCGGCAGCAATGGG
        E  D  R  G  Q  A  Q  G  G  S  D  N  D  S  D  S  G  S  N  G  488
1521    GGTGGCCAGCGGAGCCGGAGCCACAGCCGCAGCGCCAGTCCCTTCCCCAGTGGCAGCGAG
        G  G  Q  R  S  R  S  H  S  R  S  A  S  P  F  P  S  G  S  E  508
1681    CACTCGGCCCAGGAGGATGGCAGTGAAGCTGCAGCTTCTGATTCCAGTGAAGCTGATAGT
        H  S  A  Q  E  D  G  S  E  A  A  A  S  D  S  S  E  A  D  S  528
1741    GACAGTGACTGAGTCCCAGGGCATTCAGGGCTGGTTCAGACACCATTATTGTGAGCAGCA
        D  S  D  *                                                    531
1801    AAGCACTTTTCTAGTGGTCTGTTTGTGAGCCTTTCACTTGTTTGTTCCCCACCCCCAAAC
1861    CTTTGCTGTTAATAAAGTCAACTTCTCTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
1921    AAAAAAAAAAAAAAAAA
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Figure 2. Nucleotide and deduced amino acid sequence of the PD2 cDNA. The nucleotide sequence is numbered in the left-hand margin. The deduced amino acid sequence is numbered in the right-hand margin. The polyadenylation signal is underlined.

The *in vitro* translation product of PD2 mRNA, derived from the PD2 cDNA, is shown in Figure 3. The PD2 mRNA transcript was expressed by using T-7 RNA polymerase and translated in rabbit reticulocyte lysate in the presence of ^{35}S methionine. The *in vitro* translation products were analyzed by 7.5%SDS-PAGE (Figure 3). When PD2 cDNA was placed in the correct orientation, a protein of approximately 70 kDa was also seen along with three other protein bands of about 43 kDa, 44 kDa, and 45 kDa (lane 1). This pattern of reactivity was seen when non-reducing SDS-PAGE gels were run on these samples (data not shown). Similar products were not produced in lysates that contained linear pBS cut with Hind III (lane 3), PD2 in an antisense orientation, (lane 2) or lysate alone (lane 4). A positive control for *in vitro* transcription/translation analysis using the unrelated cDNA clone PD-1 (Batra *et al.*, 1991c) showed translation of a protein with the expected size of 17 kDa. The discrepancy between the observed migration of PD2 (70 kDa) and the calculated mass (59 kDa) may be either due to posttranslational modification or may be intrinsic to its amino acid sequence. It is possible that the different protein products are a consequence of cryptic T-7 promoters producing alternate transcripts from the plasmid, however sequence analysis did not reveal any evidence of these. Alternatively, these bands could be due to degradation of the translated 70-kDa protein.

Another possibility is that the observed proteins represent different folded forms of the native proteins that are not denatured by the SDS and reducing conditions we employ for these gels. This possibility is supported by an analysis of potential motifs in the primary amino acid sequence, which revealed the presence of a putative helix-loop-helix domain that may play a role in folding and dimerization of other related proteins.

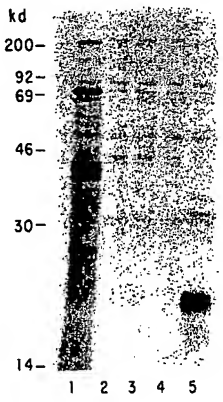


Figure 3. <u>In vitro</u> translational product of PD-2 cDNA. *Transcripts were generated from pBS using T-7 RNA polymerases, translated in rabbit reticulocyte lysate in the presence of ^{35}S methionine, and products were analyzed by SDS-polyacrylamide gel electrophoresis. Lane 1, PD2 in the sense orientation. Lane 2, PD2 in an antisense orientation. Lane 3 linear pBS cut with HindIII. Lane 4 is a control lysate containing no exogenous transcripts. Lanes 5 contain a sense construct of a previously described cDNA PD-1 (Batra et al., 1991c).*

The nucleotide sequence of PD2 did not show significant similarity to any sequence in the GENBANK or EMBL databases. However, a protein homology search through the GENBANK, EMBL, PDB and Swiss Prot databases, using BLAST and the Fasta search programs of the GCG sequence analysis software package, revealed some regions of homology to several known proteins (Table 1). Some of these regions are homologous with functionally important regions of proteins, including yeast transcriptional factor (Holmstrom *et al.*, 1994), INCENP nuclear protein (Mackay *et al.*, 1993), Glucokinase (Charles *et al.*, 1994), valyl-tRNA synthetase (Heck and Hatfield., 1988), aspartate amino transferase (Birolo *et al.*, 1991), cAMP

binding domain of the bacterial Catabolite Activator Protein (McKay *et al.*, 1982), eukaryotic regulatory type I subunit of a cAMP-dependent protein kinase (Clegg *et al.*, 1988). There is also homology with functionally important regions of several other proteins with known biological activity that are shown in the Table 1 below.

Table 1 List of proteins with partial homology to PD2.

Protein	Accession Number	Position and percentage identity
Yeast transcriptional factor PAF1	P38351	277-336, 26% in 60 residues
INCENP nuclear protein	P53352	103-192, 23%in 90 residues
Glucokinase	pdb/1glk/ 809431	243-280, 36% in 38 residues
E.coli valyl tRNA synthetase	P07118	36-133, 25% in 98 residues
RNA polymerase sigma 54 factor	P2469	68-100, 24% in 33 residues
Yeast ATP dependent RNA helicase	P15424	145-159, 47% in 15 residues
Yeast Myosin-like protein MLP1	Q02455	59-93, 37% in 35 residues
E.coli Aspartate Aminotransferase	P14909	200-270, 27% in 71 residues
Simian immunodeficiency virus reverse transcriptasen	P5896,P5897 P19509, p112502	295-326, 38%in 32 residues 318-364, 24%, 47 residues
Murine leukemia virus reverse transcriptas	P 1 1 2 2 7 , P 0 3 3 5 5 P03357	236-271, 27% in 37 residues
Recominase flp protein	P13784	300-339, 23% in 40 residues
Trypanosome RNA polymerase	P16355	313-366, 19% in 54 residues
cAMP dependent protein kinase, type 1 regulatory protein	PP12849	263-345, 23% in 83 residues
Modification methylase RSR1	P14571	312-363, 27% in 52 residues
Maternal effect protein oscar (Drosophila)	P25158	117-176, 26% in 63 residues
Inclusion body matrix protein (Viroplasmin)	P09524	125-184, 20% in 67 residues

Accession numbers are from Swissprot (P and Q), PDB, and EMBL databases. References of each of the sequence is available in the databases.

Furthermore, an analysis of the protein for potential biologically important motifs revealed the presence of a putative nuclear transport signal, a putative cAMP or related nucleotide binding site, and a putative helix-loop-helix domain that will be discussed in details later.

3.3. Chromosomal Localization

In collaboration wth Dr. James Sikela at the University of Colorado Health Science Center, the chromosomal mapping of the PD2 gene was performed using sequence-tagged-sites (STSs) as published for other cDNAs (Berry *et al.*, 1995). The 3'-untranslated region (UT) of the PD2 cDNA sequence was used to design primers for PCR of both CEPH megabase-insert YAC DNA pools, obtained from Research Genetics, Huntsville, AL (Bellanne-Chantelot *et al.*, 1992) and human x rodent somatic cell hybrid DNA (Wilcox *et al.*, 1991). The PCR-ready YAC DNA pools were constructed using a three dimensional pooling scheme which combines DNA from eight 96-well microtitre plates to give each block pool. For PCR amplification of 4 µl of YAC pool DNA was used in a 15 µl reaction with 250 um each dNTP, 1 µl of 20 µM each primer and 0.4 U of AmpliTaq polymerase in GeneAmp reaction buffer. Reactions were cycled in a Perkin Elmer Gene Amp PCR system 9600 as follows: 4 min at 94 °C; then 35 cycles of 15s at 94 °C, 1 min 15 s at 55 °C, I

min 15 s at 72 °C followed by an extension step of 10 min at 72 °C. The same set of primers were used for somatic cell hybrid panel DNA obtained from NIGMS. Approximate positions of the linkage maps were determined using the two point subroutine of the CRIMAP program package

The PD2 gene was mapped to the short arm of chromosome 19 (19p13.11-q11) by linking a YAC that PD2 mapped to (CEPH 785_d_8) out four levels to another YAC (CEPH 968_g_5). This YAC contained an STS (D19S215), which is found at Genethon position 0.38 on Chromosome 19. This position could not be translated to the cytogenetic map because chromosome 19 is not well characterized in this region. However, the STS (D19S215) has been identified on the physical map of chromosome 19. This region of the chromosome 19 is recently reported to be altered in 8 of 44 primary tumors (Griffin *et al.*, 1995). The physical location of the PD2 gene implies that amplification of this gene may be involved in the pathogensis of a subset of pancreatic adenocarcinomas.

3.4. Differential Expression of PD2

Expression of the PD2 gene was further evaluated in a panel of 14 pancreatic tumor cell lines representing various morphological stages of pancreatic cell differentiation. The putative differentiation grades for the various tumor cell lines were determined from the published morphological and ultrastructural descriptions of the cell lines and corresponding tumors and was based on the grading system of Kern (Kern *et al.*, 1987). Total RNA from these tumor cell lines were fractionated, Northern blotted and probed with PD2 cDNA. The results of this experiment are shown in Figure 4A. The PD2 cDNA probe hybridized to a message size of 1.9 kb in all cell lines, but its expression was significantly elevated only in Panc 1. Fig 4B shows rehybridization of the same filter with a B-actin probe. Over-expression of PD2 mRNA was not observed (data not shown) in breast tumor cell lines (BT 20, CAMA-1), colon carcinoma cell lines (LS 180, Colo 320), human foreskin fibroblasts (HUFF) and human B lymphocyte cell line NALM. These results suggest that the PD2 gene is overexpressed only in poorly-differentiated pancreatic tumor lines.

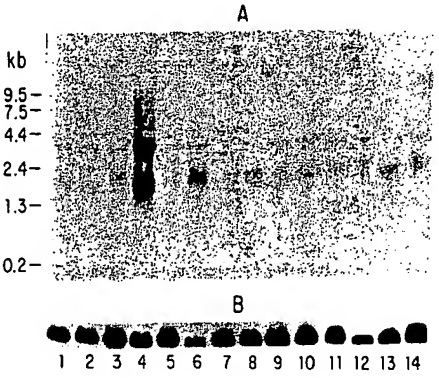


Figure 4. Northern blot analysis of total RNA (20 μg) from a panel of pancreatic carcinoma cell lines. *Total RNA were fractionated by electrophoresis on 1.2% agarose/formaldehyde gels and transferred to nitrocellulose membranes. Pancreatic carcinoma cell lines: Colo 357 (1), Fa-2C (2), Panc 89 (3), Panc 1 (4), Capan 2 (5), HS 766T (6), SW 979 (7), T3M4 (8), HPAF (9), BxPC 3 (10), AsPC 1 (11), QGP-1 (12), MiaPaCa (13) and HGC-25 (14). A was probed with PD2 cDNA; B was probed with a ß actin cDNA probe.*

3.5. Southern Blot Analysis

Purified genomic DNA from the poorly differentiated cell line Panc 1 and well differentiated cell line HPAF/CD 11 were digested with *EcoR*I, B*amH*I and *Hind*III, fractionated by agarose gel electrophoresis, Southern blotted and hybridized to the PD2 cDNA probe (Figure 5).

The probe hybridized to two or more fragments suggesting that: this gene could be part of multi-gene family; there are pseudogenes for PD2; or PD2 is a part of large gene that contained multiple restriction sites. One of the bands showed a 30-fold amplification in Panc 1 DNA as compared with HPAF/CD-11, suggesting that this is the gene encoding the transcribed product seen in Panc1 cells.

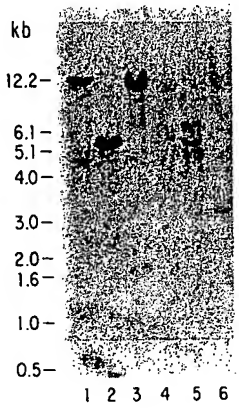
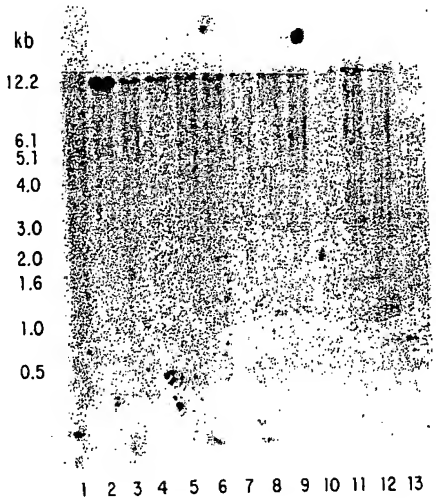


Figure 5. Southern blot analysis of genomic DNA (10 ug) from Panc l and CD11tumor lines. *After digestion with the indicated enzymes, the DNA was separated on 0.8% agarose gel and Southern blotted as per the standard methods. Lanes 1, 2 and 3 are Panc 1 DNA digested with Eco RI, Hind III and Bam HI, respectively. Lanes 4, 5, and 6 are HPAF/CD11 DNA digested with Eco RI, Hind III and Bam HI, respectively. The blot was probed with ^{32}P-labelled PD2 cDNA.*

Southern blot analysis of EcoRl-digested DNA from a large panel of tumor cell lines including six pancreatic tumor cell lines, three breast tumor cell lines, and three colon tumor cell lines confirmed that amplification of the PD2 gene occurred only in the Panc 1 cell line (Figure 6).

Figure 6. Southern blot analysis of genomic DNA from several tumor cell lines. Pancreatic cell lines: HPAF (1), Panc 1 (2), Colo 357 (3), SW 979 (4), Capan 1 (5), T3M4 (5), and HS 766T (7). Breast cell lines: CAMA-1 (8), MCF-7 (9), SkBR (10). Colon cell lines: WIDR (11), Colo 320 (12) and LS 180 (13). The blot was probed with ^{32}P-labelled PD2 cDNA.



In summary, we have identified a novel cDNA for a previously undescribed gene, PD2, whose expression in a poorly differentiated pancreatic tumor cell line, Panc 1, was 30-fold higher compared to several other pancreatic tumor cell lines, as a consequence of gene amplification. Taken together the analysis of the PD2 protein sequence for some potential biologically-important motifs (described in Figure 7 on the next page and regions of homology in Table 1) raise a spectrum of possibilities for biological activities of the PD2 protein. One possibility is that PD2 is a transcription factor with a cAMP binding site that links its activity with signal transduction pathways. Another possibility is that it is an enzyme that binds nucleotides, DNA, or RNA, or related compounds as a substrate or as a cofactor. A third possibility is that it plays a structural role in binding to RNA as a ribonucleoprotein or ribosomal component, or to DNA as a structural molecule involved in chromosomal organization.

Insertion of the Flag epitope may not interfere with expression of the PD2 Flag protein. Thus to analyze PD2 protein by another method, we have prepared polyclonal antibodies against two synthetic peptides obtained from the deduced amino acid sequence of PD2 cDNA. Two peptides were selected based on high antigenic index shown by MacVector Program Analysis.

PD2 Peptide 1 (PD2p1) region corresponding to 142-162 AA
NH2-RYGISNEKPEVKIGVSVKQQ-COOH

PD2 Peptide 2 (PD2p2) region corresponding to 327-348 AA
NH2-ETRVRLSKRRAKAGVQSGTNAL-CCOH

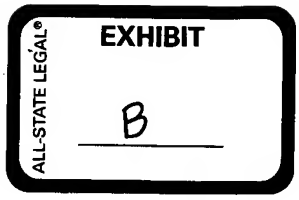
These peptides have been synthesized, conjugated to KLH and were used to immunize rabbits. Recently we analyzed the serum of one of the rabbit immunized with the PD2p2 peptide and purified anti-PD2p2 antibodies using peptide affinity chromatography. For this, an affinity column was prepared by linking PD2p2 peptide to HiTrap NHS-activated Sepharose (Pharmacia). Bound antibodies were eluted using 100 mM glycine pH 2.7 as a single peak, neutralized with 1 M Tris pH7.0 and used directly in Western blots. Equal amounts of cytoplasmic and nuclear protein lysates from Panc1 and HPAF/CD11 cell lines were loaded per lane, electroblotted to nitrocellulose and the membranes were probed with affinity purified PD2p2 antibodies. The PD2p2 antibody reacted to a band of about 70 kDa protein in Panc1 and HPAF/CD11 nuclear extracts. No bands were seen in cytoplasmic extracts of Panc1 or HPAF/CD11 cells. The intensity of PD2 protein band in the nuclear extract was 30 fold higher in Pan1 cell line as comapred to HPAF/CD11. The similar level of over-expression was seen in PD2 mRNA or gene copy in Panc1 cell line (shown in preliminary data). Affinity purified polyclonal antibodies against PD2p1 will be used in similar Western blotting analysis to confirm the observations based on Flag-tagged PD2 expression experiments.

4.1.2.2 Nuclear Localization of PD2

The PD2 protein contains a putative nuclear localization signal (KKRK) at residues 269-272 (Garcia-Butstos *et al*, 1991) and a putative arginine rich RNA binding domain (RVRLSKRRAKA) at residues 329-339 with homology to a consensus sequence (Lazinski *et al.*, 1989). In our preliminary data, Western blot analysis using anti-PD2p2 antibody showed the overexpression of PD2 protein in the nuclear extracts. Taken together putative nuclear localization signal and western blot analysis of PD2, we predict for nuclear localization of PD2.

Using confocal immuno fluorescence microscopy (available as a core facility in the Department of Biochemistry and Molecular Biology at the UNMC), the Flag-epitope-tagged PD2 transfected cells will be analyzed for subcellular localization using M2 monoclonal antibody. We will evaluate monolayer cells expressing Flag-tagged PD2 protein grown in Lab-Tek chambered slides for all three subcellular locations as described by Willingham *et al.* (1994). Following rinsing in PBS, attached cells will be fixed with 3.7% formaldehyde in PBS: first subset will be incubated in 0.1% triton X-100 in PBS and will be analyzed for intracellular nuclear staining; second subset of fixed cells will be washed with PBS-0.1% saponin and will be analyzed for detection of cytoplasmic location; third subset will be used directly for staining to assess cell-membrane staining.

The confirmation of subcellular localization of the PD2 protein will help in elucidating its potential biological functions.



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Your grant application has been received by NIH and assigned to an Initial Review Group (IRG) for scientific merit evaluation and to an Institute for funding consideration. The Initial Peer Review should be completed by [redacted] and a funding decision made shortly after the appropriate National Advisory Group meets in [redacted]. For questions about the assignment, contact the Referral Office (301) 435-0715. For questions prior to the review, contact the IRG Scientific Review Administrator (SRA) named above. For questions after the review, contact the Institute listed below.

PRINCIPAL INVESTIGATOR: BATRA, SURINDER K
TITLE: MOLECULAR STUDIES ON A NOVEL AMPLIFIED PD2 GENE
ASSIGNMENT NUMBER: 1R01CA77466-01
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IRG: PTHB

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